BIOCHIMICA ET BIOPHYSICA ACTA
BBA

The antineoplastic drug lonidamine interferes with the acidification mechanism of cell organelles

Paolo Dell'Antone *, Laura Piergallini

Dipartimento di Scienze Biomediche Sperimentali, Università di Padova, via Trieste 75, Padua, Italy

Received 16 January 1997; revised 10 March 1997; accepted 20 March 1997

Abstract

The effect of the anticancer drug lonidamine (LND) on the pH of intracellular organelles was studied in isolated rat thymocytes by fluorimetric analysis of the (bafilomycin-nigericin sensitive) uptake of the acridine orange dye (AO) into acidic compartments. LND brought about a marked reduction (> 60%) in the above pH gradients with a half maximal decrease at a concentration of 0.25 mM. LND also caused a decrease, although to a lesser extent, in the ATP levels. In isolated rat liver lysosomes, 0.6 mM LND was found to inhibit ATP-driven organelle acidification by about 80%; minor inhibition of ATPase activity was observed in the same conditions. In addition, LND was able to promote proton efflux from isolated lysosomes. On the basis of our results it is suggested that the effect of LND on intracellular proton gradients may be due partially to the decrease in ATP levels, and mostly to a drug-induced increase in the ion (proton) permeability of the membranes. © 1997 Elsevier Science B.V.

Keywords: Lonidamine; Lysosome; Proton permeability; ATP level; Acidic organelle

1. Introduction

Lonidamine (LND), a novel antineoplastic agent belongs to the class of drugs affecting cellular energy metabolism [1–5]. Based on findings in both cancer and normal cells, different biochemical functions or cell structures were proposed as the targets for the drug action; among these are the mitochondrial-bound hexokinase [2,3], components of the respiratory chain [4,5] and cell or organelle membranes ([6–8], for review [9]). The lactate transport system together with cytoplasmic pH (pH_i) and ATP levels were also recently shown to be affected by LND [10].

Using intact rat thymocytes, we found that LND significantly affected the acidification mechanism that regulates the lumenal pH of acidic cell organelles.

An analysis of this effect, using also isolated lysosomes, suggested that the decrease in the pH gradient of intracellular organelles might be linked partially to the decrease in ATP levels, and was due mostly to an increase in the ion (most likely proton) permeability of the organelle membrane.

2. Materials and methods

Rat thymocytes were prepared according to standard procedures, resuspended in a saline containing

Abbreviations: LND, lonidamine (1-(2,4-dichlorobenzyl)-1-H-indazole-3-carboxylic acid); AO, acridine orange; MDG, *N*-methyl-D-glucamine; pH_i, cytoplasmic pH; NEM, *N*-ethylmaleimide

* Corresponding author. Fax: +39 49 8276049.

PII S0167-4889(97)00049-9

135 mM NaCl 5 mM KCl, 5 mM $MgSO_4$ and 20 mM Hepes buffer pH 7.3, and maintained at room temperature. Viability was over 90% during the experiments (up to 4 hours). A lysosome-enriched preparation (8- to 10-fold) was obtained by standard centrifugations of a rat liver homogenate [11]. In some cases the suspension was further centrifuged in a continuous self-generating Percoll gradient as described [12]; the enrichment in lysosomal enzymes in these preparations was 40- to 60-fold. Protein and enzyme determinations were conducted according to procedures described in [12]. Lysosomal ATPase and proton pumping activities were measured as described [12] with minor modifications. Briefly ATPase was routinely assayed in quadruplicate by Pi release at 37°C, in the medium described below for the proton pump assay, containing, in addition, 1 mM EGTA, 200 μM sodium vanadate, 2 μg/ml oligomycin, 1 μg/ml nigericin, 1.3 mM ATP and lysosomes from a purified preparation at a final concentration of 0.1–0.2 mg/ml of protein. Other experimental details are reported in [12]. Proton pumping activity was assayed at room temperature (21 ± 2) in a medium containing 0.12 M KCl, 5 mM $MgSO_4$ and 20 mM Hepes buffer, pH 7.3 adjusted with KOH, as described [12] by following fluorimetrically ATP-driven lysosome uptake of Acridine Orange (AO). The dye was excited at 492 nm and fluorescence emission at 530 nm was collected, using a Perkin-Elmer MPF 2A spectrofluorimeter. A decrease in AO fluorescence under this conditions indicates an acidification of the organelles, due to accumulation dependent quenching. For a more detailed description of the method see [12] (and references therein).

AO uptake by acidic cell organelles was followed essentially by means of the same procedure, as previously described [13].

The relative ATP content of the cells was determined by fluorimetric measurement of $NADP^+$ reduction [14] following the addition of an ATP-releasing agent (Triton) [15], in the presence of glucose and appropriate enzyme mixture. In controls, performed by omitting one of the enzyme, hexokinase, the amount of endogenous glucose-6-phosphate was found negligible and this made possible to use routinely the above mentioned enzyme mixture. The fluorimetric signal was routinely calibrated by adding a known amount of ATP at the end of each assay; the addition of Triton or the presence of oligomycin or LND, did not interfere with the assay. When glucose was lacking in the medium, it was added at the proper concentration (5 mM) immediately before beginning the assay. The fluorimetric response was invariably recorded 3 min after ATP liberation (by Triton). Experiments were carried out at room temperature.

LND was prepared at a concentration of 0.1 M in a 0.12 M *N*-methyl-D-glucamine (MDG) aqueous solution. The LND-MDG solution was successively sonicated, stored at 4°C and used within 2 weeks. At the maximal final concentration used, neither LND nor MDG affected AO fluorescence or interfered with any enzymatic assay. pH changes in the medium were measured with a glass electrode and a Radiometer PHM 84 pH-meter connected to a Linseis recorder. All chemical reagents were purchased from Sigma Chemical Co. LND was kindly supplied by F. Angelini Institute (Rome, Italy).

3. Results

3.1. LND impairment of proton gradients in isolated thymocytes

Fig. 1 shows the AO uptake by thymocytes in different experimental conditions. In the absence of additions (Fig. 1a), AO uptake, which was complete in about 20 min, was a result of the pH gradients across the acidic cell organelles and of the activation of the proton pump [16] restoring the acidic lumenal pH (perturbed by the basic dye accumulation). The uptake of AO, on the other hand, was strongly reduced in the presence of LND (Fig. 1, trace b), which caused an average (Fig. 1, plot d) 60% dissipation of the gradient at a concentration of about 0.5 mM and half maximal dissipation at 0.25 mM. An almost identical behaviour was observed in a glucose free medium (not shown).

We then tried to investigate the mechanism of the LND-mediated inhibition of AO uptake, a complex phenomenon which might involve different cellular processes, such as decrease in ATP levels, inhibition of the proton pump, and increased proton permeability of the organelle membrane. We first analysed the effect on intracellular proton gradients of various

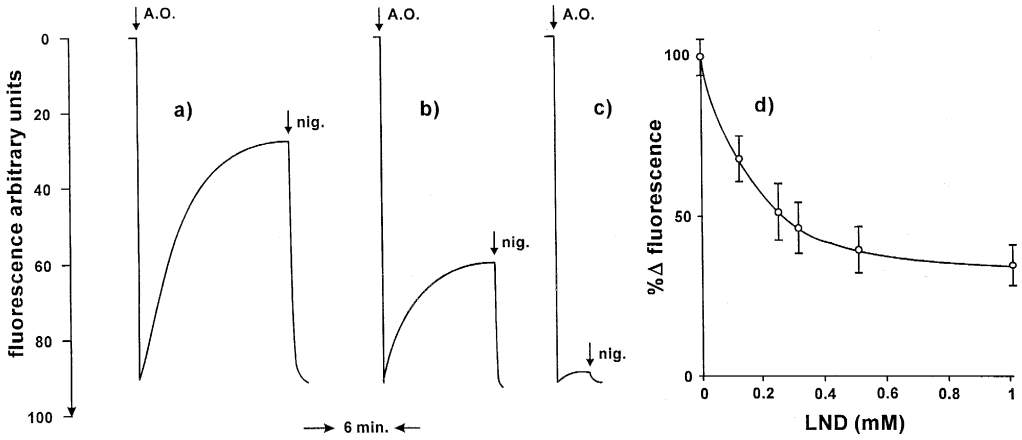

Fig. 1. Impairment of intracellular proton gradients by LND or an inhibitor of vacuolar H^+-ATPase. (a–c) Uptake of AO by rat thymocytes in the (a) absence and (b) presence of LND 0.5 mM and (c) presence of 0.3 μM bafilomycin. (d) Dependence of dye uptake on LND concentration. Data $\pm$ SD based on 10 distinct experiments. The dye accumulation into acidic vesicles of the cells was assessed by measuring dye fluorescence decrease (see Section 2). In (b) and (c), cells were incubated with 0.5 mM LND and 0.3 μM bafilomycin A1, respectively, for 20 min before receiving AO (7.5 μM, final concentration) as shown. Nigericin addition: 2 μg/ml (final concentration). The fluorescence scale and (bottom) time unit refer to all three experiments shown (a–c). In the experiments summarised in (d), cells were incubated with the indicated LND concentration for 10 min before receiving AO (7.5 μM, final concentration); 25 min later, nigericin (final concentration 2–3 μg/ml) was added and the fluorescence change recorded as shown in (a) and (b). In (d), 100% corresponds to the fluorescence change (indicating dye release) recorded in the absence of LND. Cell concentrations, 33×10^6/ml in (a–c); $25–37 \times 10^6$/ml in d).

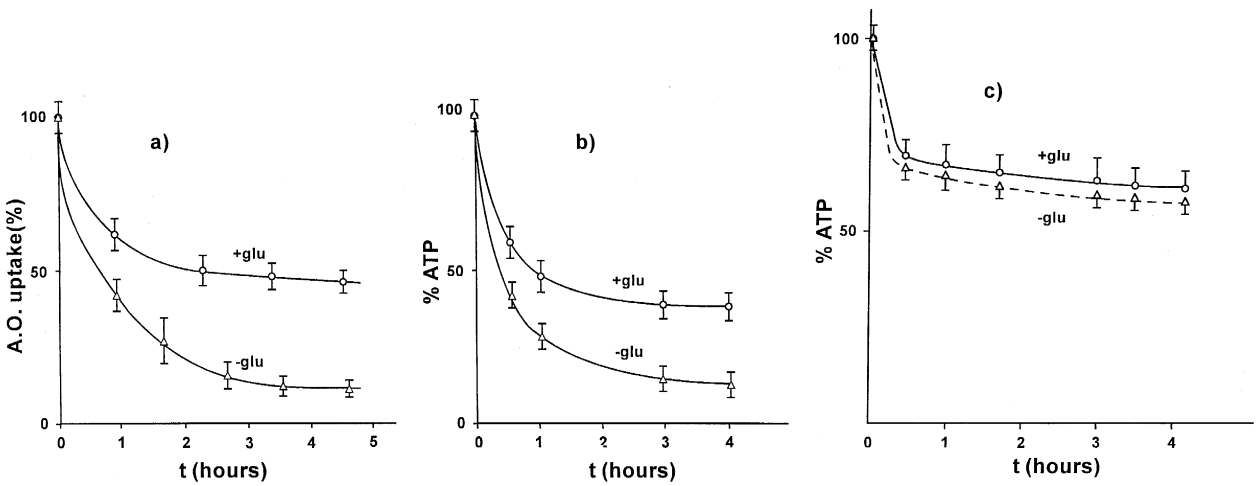

Fig. 2. Time dependence of the effect of: (a) oligomycin on AO uptake by thymocytes, (b) and (c), oligomycin and LND, respectively, on ATP levels. (a) Evaluation of the extent of AO uptake by means of the fluorescence change and other experimental details as in Fig. 1d). Data $\pm$ SD from at least 6 distinct cell preparations. (a–c) Cells, suspended in the standard (glucose) medium described in Section 2, were incubated with 1.5 μg/ml oligomycin (c) or 0.4 mM LND (a,b) for the time indicated before initiating the AO or ATP assay. In some cases glucose was omitted from the medium, as indicated. In the absence of oligomycin or LND addition, (corresponding to 100% in the diagrams) no significant variation in AO uptake or ATP levels was observed in either cases ($\pm$glucose) during the entire duration of the experiment (not more than 4 h). Similarly, oligomycin or LND had no effect in the absence of incubation (time zero). Cell concentration in (a), (b) and (c) $25–35 \times 10^6$/ml. ATP was measured as described in Section 2, following liberation of cell ATP by Triton (final concentration 0.2%).

treatments which impair the cell energy metabolism of the cells. The internal acidity of organelles of the vacuolar system is thought to be maintained by an ATP-driven H^+ pump, ([16], review [17,18]). As expected, Fig. 2a shows that oligomycin, a well known inhibitor of the mitochondrial ATP-synthetase, caused a 55% inhibition of AO uptake in the presence of glucose and a 85% in the absence following a 3–4 h incubation. Furthermore, bafilomycin A1, a specific inhibitor of vacuolar H^+-ATPase in isolated organelles [19], completely inhibited AO uptake at a concentration of 0.3 μM (Fig. 1, trace c); this finding strengthens the hypothesis that AO uptake is driven by the proton gradient across the membrane of acidic organelles.

As expected, oligomycin not only reduced AO uptake, but it also affected ATP levels in a similar manner (Fig. 2b); bafilomycin A1, on the other hand, did not modify ATP levels (not shown). LND caused a decrease in ATP levels (Fig. 2c), which, unlike for oligomycin, was not significantly different in the presence and in the absence of glucose; most of the decrease occurred after 30 minutes incubation. Furthermore, the LND-induced reduction in ATP levels was less than that required (on the grounds of the findings with oligomycin, see Fig. 2) to induce the decrease in the pH gradients elicited by LND. This suggested that, in addition to ATP levels, other factors could be involved in the LND impairment of proton gradients.

3.2. LND effects on ATP driven acidification, ATPase activity and proton permeability in isolated lysosomes

A striking inhibition of the H^+-pump was induced by LND in isolated rat liver lysosomes (compare traces a and b of Fig. 3), which was almost complete (80%, on the average) at 0.6 mM drug (Fig. 3, plot d). Trace c also shows that 50 nM bafilomycin A1 completely inhibited ATP–driven AO uptake. To test whether the LND effect might be due to a drug-induced proton permeability that would also result in an apparent inhibition of the proton translocating mechanism, we took advantage of the finding that the pH gradient is, at least in part, conserved for some time in freshly prepared, lysosome-enriched suspensions, maintained in a (K^+-free) sucrose medium [11]. As shown in Fig. 4 (trace a and b), where higher lysosome concentrations were used compared to Fig.

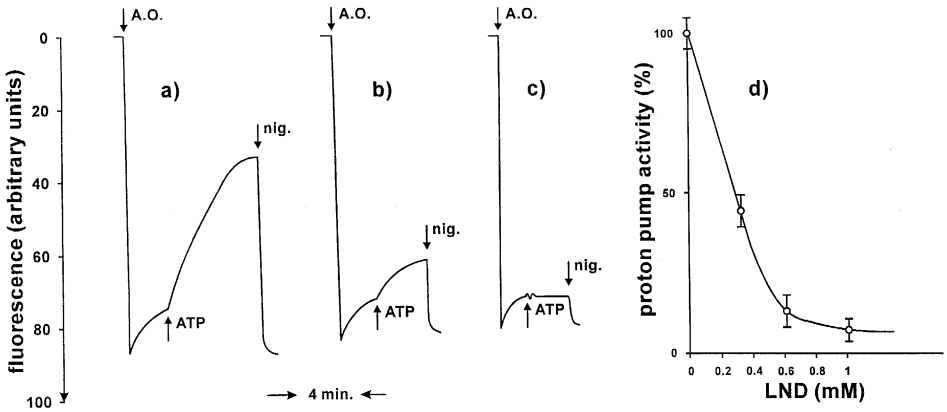

Fig. 3. LND or bafilomycin A1 inhibition of ATP-driven lysosome acidification. In (a–c), lysosome-enriched preparations were used. The low AO uptake before ATP addition is due to a resistant small pH gradient in these preparations. In (b) LND 0.4 mM was present. In d) the extent of ATP-induced AO fluorescence change (percent of the value recorded in the absence of the drug) is shown as a function of LND concentration. In (b) and (d) the drug was added 10 min about before AO (data $\pm$ SD from 7 different lysosomes preparations including purified lysosomes). In (a–c) final concentrations of AO, ATP and nigericin were 5 μM, 1 mM and 1 μg/ml, respectively. The time scale indicated at the bottom refers to all (a–c). In (c), bafilomycin A1 was added (50 nM, final concentration) a few minutes before AO. The protein concentration of the lysosomal suspension was 0.3 mg/ml in (a–c), and 0.2–0.4 and 0.05–0.1 mg/ml in (d) when enriched and purified lysosome preparations, respectively, were used. Since no significant difference in the inhibition curve was observed (the dye responds only to organelles able to acidify their interior) when enriched and purified lysosome preparations were used, data from two preparations could be used together to construct the curve shown in (d).

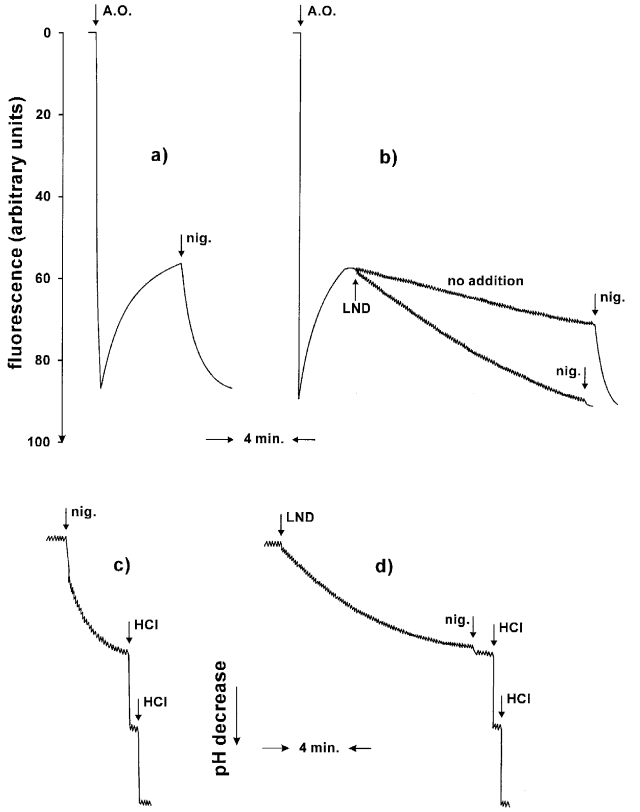

Fig. 4. (a,b) Dissipation of the lysosomal pH gradient by nigericin or LND; (c,d) medium acidification following addition of nigericin or LND. An aliquot (50–100 μl) of a lysosome-enriched suspension in a sucrose medium was added to 2 ml of a KCl medium immediately before AO uptake or pH determination, respectively. AO concentration in (a,b) 5 μM. Nigericin and LND addition (final concentrations): 1 μg/ml and 0.5 mM in (a,b) respectively, the double in (c,d). HCl addition in (c) and (d), 2 μl from a 0.1 M HCl solution. Medium buffer concentration: 20 mM in (a) and (b); 6 mM in (c) and (d). Protein concentration was in (a) and (b) = 0.7 mg/ml in (c) and (d) = 1.4 mg/ml. The experiments shown were repeated at least three times using different preparations, and similar results were obtained.

3, the pH gradient was unmasked by the AO uptake which could be recorded in the absence of ATP. Due to the high K^+ medium used in these assays, the gradient was obviously destroyed by nigericin [11]. A similar albeit slower effect was observed following LND addition. In the absence of additions (Fig. 4, trace b), there was a time dependent loss of pH gradient which, however, was significantly slower (about 1/3, when comparing initial rates) than that elicited by LND. The proton efflux inferred by the above results was directly measured as a pH decrease in the external medium in a poorly buffered medium (Fig. 4, trace c and d).

In both cases (nigericin or LND addition), the time courses of AO fluorescence and of pH changes in the medium were very similar, as shown in the same figure.

Finally, we analysed the effect of LND on ATPase activity in purified lysosomes. To prevent the masking of ATPase inhibition by the LND-induced increase in proton permeability, which is thought to stimulate ATPase activity [12], the assays were performed in the presence of nigericin. Some LND inhibition was observed (24% with SD 8% ($n = 6$), data not shown); however, when the measurements were repeated in the presence of NEM or bafilomycin, ATPase inhibition by LND was stronger and the increase was comparable to that of LND alone. Thus, the significance of these data remained unclear, as discussed below.

4. Discussion

In confronting the problem of an anticancer drug's mechanism of action it is evidently important to study its effect on normal cells.

Many cellular activities or structures have been proposed as the targets of LND action [1–8]. Recently, lactate, ATP levels and pH_i were measured in LND-treated cultured human breast cancer cells by means of ^{31}P-NMR; lactate was found to accumulate inside the cells, while ATP levels and pH_i were both decreased; LND did not inhibit hexokinase activity (assayed by means of ^{13}C-NMR), thus suggesting that glycolysis was poorly affected by LND [10]. However, in contrast with these data, the inhibition of hexokinase by LND in cultured human cancer cells has also been recently reported [20]. This discrepancy might arise from the different experimental conditions (intact cells in ref. [10], versus lysed cells in [20]). We also observed a reduction in the levels of ATP following drug addition. Moreover, at concentrations not much higher than those tested in other studies, we observed a significant decrease in the acidity of the intracellular organelles.

The comparison of the effects of LND on the acidity of the intracellular organelles and on the levels of ATP suggests that the drop in pH gradients could be partially explained by the decrease in the ATP levels; however, the LND-induced H^+-pump impairment, that we could observe in isolated organelles, needs to be taken into account. Our results suggest two hypothesis for this effect: (i) an increased H^+ (or H^+-K^+) conductance of the organelles membranes; this is shown in our study by two independent findings, i.e., LND induced both AO and H^+ efflux from lysosomes; (ii) an impairment of the enzymatic activity that drives proton pumping. However, the interpretation of results concerning ATPase activity is not straightforward. In fact, the LND-inhibitable ATPase fraction did not completely overlap with the NEM (or bafilomycin)-sensitive ATPase. On the other NEM-sensitive ATPase is only a fraction of the overall ATPase activity ([12], and references therein, [21]) and even in highly purified lysosomal preparations, minor contamination by other subcellular fractions cannot be ruled out [12]. The inhibition provided by LND could not thus be linked to proton pumping but involve other ATPase activities and further investigation is needed.

The rapid drop (20 min of incubation) in the pH gradient observed following the addition of the vacuolar H^+-ATPase inhibitor, bafilomycin A1 (Fig. 1, trace c), might in part reflect a modification in lumenal pH due to AO uptake into acidic compartments; however, this finding seems also to indicate that a continuous enzyme activity is required to maintain the pH gradients, probably to overcome a proton leakage from the organelles (or equivalent transport process also resulting in internal pH increase), as suggested also by the data of Fig. 4 (trace b). Although we observed this proton flux in isolated organelles when proton discharge was caused by nigericin or LND, whether it is sufficient to elicit the LND-induced decrease in pH_i observed by others [10], is a moot point.

Our finding of a decrease in ATP levels is in line with the current notion that LND affects the energy production mechanism; however, in our case (see also [10]), it cannot be explained by an inhibition of the glycolytic pathway alone, because ATP depletion by the drug was observed also in the absence of glucose (Fig. 2c), and glucose shortage has no or very little effect on the ATP levels for some hours in these and other normal cells [15,22]. Similarly, the effect of LND on the intracellular proton gradient was observed also in the absence of glucose, thus suggesting that it could not be linked to glycolysis inhibition. Therefore, it is possible that an impairment in respiration linked energy production is involved, and, although indirectly, our findings (see also [5]) suggest the increase in proton permeability as a drug effect that could impair the energy-producing apparatus of the mitochondria. In contrast with this suggestion [for a more complete discussion see [5]), early studies on the LND effect indicated that it inhibited respiration as well as lactate production in intact cells [23]. However, it was recently reported that in isolated myocytes, under conditions in which cytochrome oxidase turns over at its maximal rate, the rate of oxygen consumption was increased when the cells were obtained from LND treated rats [5] and in an another recent study [20] the percent of coupled respiration was found to decrease after exposure of human cancer cells to LND.

Finally, it should be noticed that LND effects were not superimposible to those of oligomycin. Thus, an another possibility that should be considered is that LND induces energy overconsumption (due possibly to proton cycling) more than impairing energy producing processes.

In conclusion, the present study confirms that the action mechanism of the antineoplastic agent LND may involve multiple effects, among which a decrease in the acidity of organelles of the vacuolar system, partially linked to a decrease in ATP levels, but, in our view, mostly to an increased membrane ion (most likely proton) permeability. It must be observed that the drug concentrations necessary to induce maximal effects in our study are higher (about double) than in others (see, e.g. [20]). However preliminary findings in Ehrlich ascites tumor cells (paper in preparation) show similar effect at concentrations 2–3 times lower. Thus, the possibility that LND utilises the above or other correlated events to exert its damaging effect on cancer cells, by possibly taking advantage of their physico-chemical or biochemical features (such as different pH_i [24] and energy metabolism [25] and lower capacity to maintain normal ATP levels in the face of energy substrate shortage [15,22,26]), would deserve further investigations.

References

[1] B. Silvestrini, G. Palazzo, M. de Gregorio, Prog. Med. Chem. 21 (1984) 111–135.
[2] A. Floridi, M.G. Paggi, M.L. Marcante, B. Silvestrini, A. Caputo, C. de Martino, J. Natl. Cancer Inst. 66 (1981) 497–4999.
[3] A. Floridi, M.G. Poggi, S. D'Altri, C. de Martino, A. Caputo, Cancer Res. 41 (1981) 4661–4666.
[4] A. Floridi, A. Lehninger, Arch. Biochem. Biophys. 226 (1983) 73–83.
[5] P. Sarti, G. Antonini, G. Arancia, J.B. Thomas, G. Citro, A. Melomi, A. Molinari, F. Malatesta, Biochem Pharmacol 12 (1994) 2221–2225.
[6] W. Malorni, G. Arancia, C. de Martino, A. Nista, L. Accinni, R. Masella, A. Cantafora, Exp. Mol. Pat. 49 (1988) 361–372.
[7] M.T. Santini, C. Cametti, P. Indovina L, W. Malorni, M. Benassi, F.P. Gentile, S. Castiglione, A. Floridi, FEBS 291 (1991) 169–172.
[8] W. Malorni, S. Meschini, P. Matarrese, G. Arancia, Anticancer Res. 12 (1992) 2037–2046.
[9] B.A. Teicher, Eur. J. Cancer 30 A (1994) 1411–1413.
[10] H. Ben-Horin, M. Tasini, A. Vivi, G. Navon, O. Kaplan, Cancer Res. 55 (1995) 2814–2821.
[11] P. Dell'Antone, FEBS Lett. 160 (1984) 15–22.
[12] P. Dell'Antone, Arch. Biochem. Biophys. 262 (1988) 314–325.
[13] P. Dell'Antone, J. Cell Phys. 139 (1989) 76–82.
[14] J.R. Williamson, B.E. Corkey, Methods Enzymol. XIII (1969) 434–513.
[15] P. Dell'Antone, FEBS Lett. 350 (1994) 183–186.
[16] P. Dell'Antone, Biochem. Biophys. Res. Commun. 86 (1979) 180–189.
[17] I. Mellman, R. Fuchs, A. Helenious, Annu Rev. Biochem. 55 (1986) 663–700.
[18] G. Rudnick, Annu. Rev. Physiol. 48 (1986) 403–413.
[19] E.J. Bowman, A. Siebers, K. Altendorf, Proc Natl. Acad. Sci. 85 (1988) 7972–7976.
[20] G. Ara, B.A. Teicher, Int. J. Oncology 8 (1996) 865–873.
[21] I. Sabolic, D. Brown, J.M. Verbavatz, J. Kleinman, Am. J. Physiol. 266 (1994) F868–F877.
[22] G.Ev. Demetrakopoulos, B. Linn, H. Amos, Cancer Biochem. Biophys. 6 (1982) 65–74.
[23] C. De Martino, T. Battelli, M.G. Paggi, A. Nista, M.L. Marcante, S. D'Atri, W. Malorni, M. Gallo, A. Floridi, Oncology 41 (1984) 15–29.
[24] I.F. Tannock, D. Rotin, Cancer Res. 49 (1989) 4373– 4384.
[25] O. Warburg, Science 123 (1956) 309–314.
[26] G.S. Karczmar, J.M. Arbeit, B.J. Toi, A. Speder, M.W. Weiner, Cancer Res. 52 (1992) 71–76.